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In yeast, Ca^{2+} and octylguanidine interact with porin (VDAC) preventing the mitochondrial permeability transition

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Abstract

In yeast, Ca^{2+} and long chain alkylguanidines interact with mitochondria modulating the opening of the yeast mitochondrial unspecific channel. Mammalians possess a similar structure, the mitochondrial permeability transition pore. The composition of these pores is under debate. Among other components, the voltage-dependent anion channel has been proposed as a component of either pore. In yeast from an industrial strain, octylguanidine and calcium closed the yeast mitochondrial unspecific channel. Here, the effects of the cations Ca^{2+} or octylguanidine and the voltage-dependent anion channel effector decavanadate were evaluated in yeast mitochondria from either a wild type or a voltage-dependent anion channel deletion laboratory strain. It was observed that in the absence of voltage-dependent anion channel, the yeast mitochondrial unspecific channel was desensitized to Ca^{2+}, octylguanidine or decavanadate but remained sensitive to phosphate. It is thus suggested that in yeast mitochondria, the voltage-dependent anion channel has a cation binding site where Ca^{2+} and octylguanidine interact, conferring cation sensitivity to the yeast mitochondrial unspecific channel.

Keywords: Ca^{2+}; Permeability transition; Porin; Yeast mitochondria; YMUC

1. Introduction

The mitochondrial permeability transition is defined as the sudden increase in mitochondrial conductance due to the opening of an unspecific, mildly anionic pore. The permeability transition is associated to a pore which exhibits a molecular mass cutoff of up to 1.5 kDa in mammals [1] and 1.1 kDa in yeast [2]. Patch clamp studies demonstrated the presence of ion channels in the internal mitochondrial membrane. The conductance range of these channels may be near 1 nS both in yeast and in mammals [3–5]. The mammalian mitochondrial 1 nS channel and the permeability transition pore (PTP) exhibit similar sensitivity to different effectors such as Cyclosporine A (CsA), further indicating that these two structures are the same [6].

The composition of these pores is still debated, i.e., a large body of evidence has been presented in favor or against the participation of the adenine nucleotide carrier (ANC) from the inner mitochondrial membrane [7] and the voltage-dependent anion channel (VDAC) from the outer mitochondrial membrane [8] in the pore. Other accessory proteins such as cyclophilin D, and specific phospholipids may be part of this structure [9,10]. It has been suggested that the pore is not a stable structure traversing both the inner and the outer membrane, but instead in the open state it permeates only the internal membrane, i.e., the structure is not assembled. In contrast, when closed, the whole VDAC/ANC complex assembles [11,12]. By contrast, porin may open independently

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allowing flux of different metabolites between the intermembrane space and the medium [12].

In yeast, the existence of a mitochondrial unspecific pore has been reported [13,14]. This yeast PTP (yPTP) [2] also termed yeast mitochondrial unspecific channel (YMUC) [15] exhibits a similar cutoff size to that of the mammalian PTP, is active in situ and is inhibited upon ATP depletion [16]. However, in regard to the structure of the YMUC, when the genes encoding either the VDAC or the ANC isoforms have been deleted, the pore is still detected suggesting that at least in yeast, other proteins different from the ANC and VDAC may constitute this pore [17]. One such candidate is the phosphate carrier, which belongs to the same mitochondrial 6- transmembrane domain family of transporters [18].

In mammalians, Ca^{2+} seems to interact with an external site where it closes the PTP while an internal site promotes the permeability transition [19,20]. In yeast, Ca^{2+} promotes closure of the pore [21]. In both cases, the Ca^{2+} concentration used is above the physiological Ca^{2+} concentrations encountered [20,21]. However, the experiments in yeast may still be meaningful as Ca^{2+} is known to interact synergistically with phosphate and Mg^{2+} [21]. Furthermore, in the presence of submicromolar concentrations of Ca^{2+}, reconstituted VDAC exhibits different conductance states [22]. In the mouse, VDAC residues E72 and E202 are located on two different cytosolic loops, which may interact with Ca^{2+} [23,24].

It is reported that alkylguanidines, which are amphiphilic cations, inhibit the permeability transition in heart mitochondria [25,26]. At relatively high concentrations, alkylguanidines also inhibit state-3 mitochondrial respiration in rat liver mitochondria [27] mainly as a result of ADP phosphorylation inhibition [28]. In yeast, octylguanidine (OG) has been reported to be an effective inhibitor of K^+ transport [29]. It has been speculated whether the interaction site of alkylguanidines in mammalian mitochondria could be a negative vestibule created by some of the 15 glutamate and 6 aspartate residues in the ANC [25]. However, further experiments are required to conclude on the possible involvement of the translocase in the OG-mediated PTP closure. Additionally, OG protects the heart during ischemia–reperfusion both in vivo and in vitro, and it has been proposed that cardio-protection is the result of the inhibition of the mitochondrial permeability transition [26]. In regard to the yeast mitochondrial unselective channel (YMUC), we observed that addition of either hexylguanidine or OG prevented the permeability transition. For OG, an inhibitory dose one magnitude lower than in mammalian mitochondria was obtained [21,25].

In contrast to Ca^{2+}, the OG-mediated inhibition of the YMUC is not reverted by depletion of the transmembrane potential as evidenced in experiments measuring contraction of swollen yeast mitochondria [21]. In order to define the yeast mitochondrial site for interaction with Ca^{2+} and OG, it was decided to test the effect of these cations on two laboratory strains, a wild type and a VDAC deletion mutant. In addition, the VDAC modulator decavanadate (dVO_4) was used as a control. Our results indicate that, in the absence of VDAC, the YMUC is desensitized to Ca^{2+}, OG and dVO_4, but not to inorganic phosphate. Thus, it is suggested that VDAC contains a Ca^{2+}/OG binding site which promotes closure of the YMUC.

2. Materials and methods

2.1. Materials

All chemicals were reagent grade. Mannitol, MES, Triethanolamine (TEA), orthovanadate, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), safranine-O, KCl, $CaCl_2$ and bovine serum albumin (BSA) type V were purchased from Sigma Chem. Co. (St. Louis, MO). All other reagents were of the highest purity available. The baker's yeast *Saccharomyces cerevisiae* laboratory strains were kindly provided by Dr. Fred Sherman (University of Rochester School of Medicine and Dentistry, New York) [30]. Alkylguanidines were synthesized by Dr. Antonio Peña (IFC, UNAM).

2.2. Laboratory strain characteristics

1) B-7553 (Wild Type): MATa $CYC1^+$ *cyc7*-738::CYH2 *ura3*-52 *his3*-Δ1 *leu2*-3, 112*trp1* 289 *cyh2*.
2) B-10610 (Δpor1): MATa $CYC1^+$ *cyc7*-738::CYH2 *ura3*-52 *his3*-Δ1 *leu2*-3, 112*trp1* 289 *cyh2 por1*-Δ::TRP1.

These strains have been used in a previous study where it was demonstrated that the growth of B-10610 was hypersensitive to high KCl in the growth medium while the B-10611 mutant lacking por2 was similar to the wild type [30]. This strengthens the concept that porin2 is not a channel [31].

2.3. Isolation of yeast mitochondria

This was performed as described previously [32]. Briefly, yeast were incubated in YPD preculture medium at 30 °C, under agitation at 250 rpm for 24 h and subsequently cultured in YPlac medium (1% yeast extract, 1% KH_2PO_4, 12% $(NH_4)_2SO_4$, 2% lactic acid, pH 5) at 250 rpm, 30 °C for approximately 24 h until they reached an optical density of 3–3.5 at 660 nm. Cells were washed and resuspended in preincubation buffer for 15 min (0.1 M Tris, 0.5 M β-mercaptoethanol, pH 9.3). Cells were washed with KCl buffer (0.5 M KCl, 10 mM Tris pH 7.0) to remove excess β-mercaptoethanol and then these were resuspended in digestion buffer (1.35 M sorbitol, 1 mM EGTA, 10 mM citric acid, 10 mM NaH_2PO_4, 10 mM $NaHPO_4$, pH 5.8), and zymoliase was added at 10 mg/g cell dry weight. Cell lysis was measured at 600 nm in a Beckman DU-50 spectrophotometer [33]. Cells were washed in protoplast buffer (0.75 M sorbitol, 0.4 M mannitol, 10 mM Tris, 0.1% BSA, pH 6.8), resuspended in homogenization buffer (0.6 M mannitol, 2 mM EGTA, 10 mM Tris, 0.2% BSA, pH 6.8) and lysed using a Potter homogenizer and a tight pestle using 20 passes. From the homogenate, mitochondria were isolated by differential centrifugation in a SS34 Sorvall rotor, frozen in a Revco freezer at −70 °C and thawed prior to each experiment [32]. Protein concentration was determined by the biuret method [34]. Control experiments were conducted in non-frozen mitochondria from each strain and the effects of Ca^{2+}, OG and dVO_4 were the same as in frozen/thawed mitochondria (results not shown).

2.4. Oxygen uptake

The oxygen consumption rate was measured in resting state (State 4) and in the presence of the uncoupler CCCP (State U), using a YSI model 5300 Oxygraph equipped with a Clark electrode in a 3 mL water jacketed chamber (at 30 °C) containing mitochondria at a final concentration of 0.5 mg protein/mL [35]. The reaction mixture was 0.6 M mannitol, 5 mM MES, pH 6.8 (TEA), 20 mM KCl plus 5 μL/mL ethanol as respiratory substrate. The concentrations of Pi, Ca^{2+} and K^+ used are indicated in the legends to figures and tables. Stock solutions were 2.0 M KCl, 1.0 or 0.1 M Pi–Tris, pH 6.8. K^+ was used to promote swelling, to modulate respiration or to deplete the transmembrane potential as a result of its electrophoretic uptake through the open YMUC [21,30]. These effects do not occur on mannitol alone [13].

2.5. Transmembrane potential (ΔΨ)

The ΔΨ was determined spectrophotometrically using a DW2000 Aminco spectrophotometer in dual mode. The reaction mixture was 0.6 M mannitol, 5 mM MES, pH 6.8, 20 mM KCl plus 5 μL/mL ethanol and 10 μM safranine-O, absorbance changes were followed at 511–533 nm [36].

2.6. Mitochondrial swelling

The K^+-mediated swelling was determined at room temperature. The reaction mixture was 0.6 M mannitol, 5 mM MES, pH 6.8 (TEA), plus 5 μL/mL ethanol. Swelling was promoted adding 20 mM KCl as indicated. The absorbance changes were measured at 540 nm in a DW 2000 Aminco spectrophotometer in split mode equipped with a magnetic stirrer [37].

3. Results

3.1. Ca^{2+} sensitivity of mitochondria from a Δpor1 yeast strain

In the field of mammalian mitochondria, there is a heated controversy on the location of the Ca^{2+} binding sites within the PTP. VDAC seems to possess binding sites for the cation [12,23,24]. In addition, Ca^{2+} seems to interact with cyclophilin D [38,39] and it has been demonstrated that porin-null mice exhibit increased resistance to Ca^{2+}-induced cell death [40]. In yeast, this problem has not been addressed, although Ca^{2+} does promote closure of the YMUC [21]. Thus, we decided to test the sensitivity to both Ca^{2+} and OG in mitochondria from a *S. cerevisiae* VDAC deletion strain (Δpor1). These studies seemed more interesting as it has been shown that the YMUC is functional in VDAC-less yeast mitochondria [17].

The effects of Ca^{2+} on isolated mitochondria from the WT and the Δpor1 strains were analyzed in oxygen consumption experiments (Table 1). Both strains retained the sensitivity to phosphate, which has been shown to promote closing of the YMUC [13–15,21], probably at a site located in the matrix side of the inner membrane [15,41]. In agreement with previous reports using other WT strains [13–15,21], the WT exhibited a U/4 quotient of 2.2 at 4 mM Pi which decreased down to 1.0 at 0.4 mM Pi. In the presence of low Pi, the U/4 quotient increased in the presence of increasing concentrations of Ca^{2+} as follows: in the presence of 0.2 mM, Ca^{2+} U/4 was 1.4 and at 0.4 to 1.0 mM Ca^{2+}, it increased to 1.6–1.7. The Δpor1 strain exhibited the same response to Pi as the wild type, i.e., at 4 mM Pi U/4 was 1.7 while at 0.4 mM U/4 was 1.1. However, the Δpor1 strain was not sensitive to Ca^{2+}, as indicated by the U/4 quotients of 1.1 observed at all the Ca^{2+} concentrations tested (Table 1).

Table 1
Effect of Ca^{2+} on the oxygen consumption rate of mitochondria isolated from a wild type and a Δpor1 strain of yeast

[P1] (mM)	[Ca^{+2}] (mM)	Wild type			Δpor1		
		Rate in U state	Rate in State 4	U/4	Rate in U state	Rate in State 4	U/4
4.0	0	396±15	180±13	2.2	406±10	238±12	1.7
0.4	0	306±12	306±11	1.0	365±8	331±11	1.1
0.4	0.2	300±10	218±10	1.4	362±10	328±9	1.1
0.4	0.4	309±9	193±12	1.6	366±11	322±13	1.1
0.4	0.6	306±12	180±9	1.7	353±12	327±13	1.1
0.4	1.0	313±11	196±10	1.6	360±10	321±10	1.1

Experimental conditions: 0.6 M mannitol, 5 mM MES, pH 6.8, 20 mM KCl, pH 6.8 (TEA), 5 μL ethanol/mL. Where indicated, 4 or 0.4 mM Pi–Tris, Ca^{2+} as indicated in the second column. To obtain the uncoupled state, 6 μM CCCP (trifluoromethoxycarbonyl-cyanide phenylhydrazone) was added. Measurements were conducted in a water-jacketed chamber (30 °C) connected to an oxymeter interfaced to a computer. Rates of oxygen consumption in the resting state (4) and in the uncoupled state (U) are expressed in natoms gram O (min mg prot)$^{-1}$. U/4 is the result of dividing the rate in the uncoupled state over state 4 in each condition. Isolated mitochondria were used at a final concentration of 0.5 mg prot/mL. Each point represents the mean of three experiments±standard deviation.

In order to further explore the effect of Ca^{2+} on the permeability of isolated mitochondria from the WT or the Δpor1 strains, the mitochondrial ΔΨ was measured in the presence of different concentrations of Ca^{2+}. Both the WT and Δpor1 mitochondria were sensitive to Pi, exhibiting a high, stable ΔΨ at 4 mM Pi (Fig. 1A and B, traces a) and a low unstable ΔΨ at 0.4 mM Pi (Fig. 1A and B, traces b). In contrast, the response to Ca^{2+} was different in these strains. In WT strain mitochondria, the ΔΨ increased proportionally with [Ca^{2+}], and at either 0.5 or 0.6 mM Ca^{2+}, it became stable and almost as high as in mitochondria exposed to high Pi (Fig. 1A, traces c–g). The ΔΨ of the Δpor1 strain mitochondria was only marginally sensitive to Ca^{2+} as it was not established at any of the Ca^{2+} concentrations tested (Fig. 1B, traces c–g). The partial effect of Ca^{2+} in the Δpor1 strain probably results from effects of Ca^{2+} at other sites, e.g., the Mg^{2+}-sensitive K^+ uptake system [42].

In order to test the permeability to K^+ of mitochondria from both the WT and the Δpor1 strains, and the Ca^{2+}-mediated control of this permeability, the swelling rate was measured in the presence of different Ca^{2+} concentrations. In agreement with the oxygen consumption and the ΔΨ results, swelling was inhibited in both strains by 4 mM Pi (Fig. 2, trace a) but not by 0.4 mM Pi (Fig. 2, trace b). In regard to the Ca^{2+} sensitivity, the swelling rate of WT mitochondria decreased proportionally with [Ca^{2+}] reaching an inhibition similar to that observed at high Pi (Fig. 2A, traces c–f). In contrast, the Δpor1 strain exhibited a high rate of swelling regardless of the Ca^{2+} concentration (Fig. 2B, traces c–f). These results of oxygen consumption, ΔΨ and swelling suggested that in WT mitochondria the YMUC was closed by Ca^{2+} which is in agreement with previous results [21]. In contrast, in mitochondria from the Δpor1 strain, the sensitivity of the YMUC to Pi was retained while the effect of Ca^{2+} was lost.

3.2. Effect of octylguanidine (OG) and decavanadate (dVO_4) on mitochondria from a Δpor1 yeast strain

The amphiphilic cation OG is capable of closing both the mammalian PTP and the yeast YMUC [21,25]. Thus, OG may be a useful tool to study the properties of either pore. In addition, the OG interaction with the pore is more stable than that of Ca^{2+}, as suggested by the fact that it promotes closure of the YMUC even in swollen yeast mitochondria [21]. In this sense, no data exist on whether OG and Ca^{2+} interact with

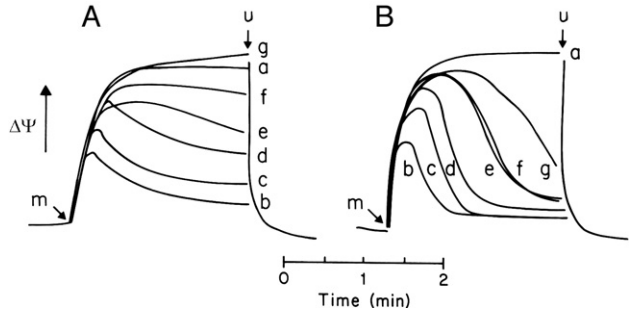

Fig. 1. Effect of Ca^{2+} on the mitochondrial transmembrane potential of *S. cerevisiae* mitochondria isolated from a wild type and a Δpor1 strain. Experimental conditions: as in Table 1, except 10 μM safranine-O. Absorbance was measured at 511–533 nm in a DW2000 (Olis-updated) Aminco spectrophotometer in dual mode. (A)=mitochondria from the wild type strain, (B)=mitochondria from the Δpor1 strain. Traces in both panels A and B were: a=4 mM Pi, no Ca^{2+}, b=0.4 mM Pi no Ca^{2+}, c=0.4 mM Pi plus 0.1 mM Ca^{2+}, d=0.4 mM Pi plus 0.2 mM Ca^{2+}, e=0.4 mM Pi plus 0.4 mM Ca^{2+}, f=0.4 mM Pi plus 0.5 mM Ca, g=0.4 mM Pi plus 0.6 mM Ca^{2+}. Representative experiment from $n=3$.

equivalent sites in the PTP or in the YMUC. Thus, we decided to test the effect of OG on the oxygen consumption rates by mitochondria from either the WT or the Δpor1 strains. It was observed that 12.5 μM OG increased the U/4 quotient in the WT mitochondria from 1.0 to 1.6 while in the Δpor1 mitochondria OG had no effect on the U/4 quotient (Table 2). In addition, dVO_4 was tested. It has been reported that dVO_4 closes the YMUC possibly interfering with purine–nucleotide binding

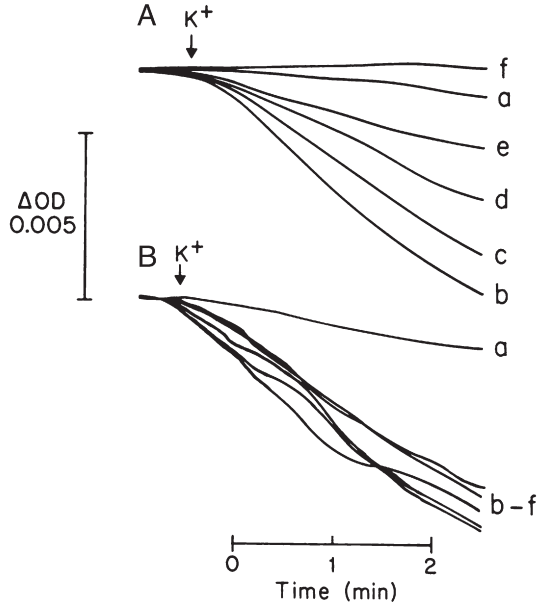

Fig. 2. Effect of Ca^{2+} on the swelling rate of mitochondria isolated from a wild type and a Δpor1 strain of yeast. Experimental conditions: as in Table 1, except no KCl was added in the medium. Absorbance was measured at 560 nm in a DW2000 (Olis-updated) Aminco spectrophotometer in split mode. (A)=wild type, (B)=Δpor1. In both panels A and B, traces were: a=4 mM Pi, no Ca^{2+}, b=0.4 mM Pi no Ca^{2+}, c=0.4 mM Pi plus 0.1 mM Ca^{2+}, d=0.4 mM Pi plus 0.2 mM Ca^{2+}, e=0.4 mM Pi plus 0.4 mM Ca^{2+}, f=0.4 mM Pi plus 0.6 mM Ca^{2+}. K^+ indicates the addition of 20 mM KCl. Representative experiment from $n=3$.

Table 2
Effect of OG or decavanadate on the rate of oxygen consumption in mitochondria isolated from a wild type and a Δpor1 strain of yeast

Pi (mM)	Wild type			Δpor1		
	Rate in U state	Rate in State 4	U/4	Rate in U state	Rate in State 4	U/4
4	377±7	185±6	2.0	398±12	253±12	1.6
0.4	326±4	311±18	1.1	344±11	344±11	1.0
0.4+OG	328±13	204±9	1.6	338±10	338±10	1.0
0.4+DV	302±15	211±5	1.5	313±16	313±16	1.0

Experimental conditions: as in Table 1, except 12.5 μM OG or 10 μM decavanadate (DV) was added where indicated. $n=3$, data±standard deviation.

sites; thus testing the effect of this polyanion seemed interesting [17]. In WT mitochondria, dVO_4 promoted an increase in the U/4 quotient to 1.5 while it did not have any effect on mitochondria from the Δpor1 strain (Table 2).

In order to further explore the effect of OG and dVO_4 on the YMUC, the mitochondrial ΔΨ was measured under the following conditions: in the presence of 4 mM phosphate, where a high transmembrane potential was established both in the WT and in the Δpor1 strain (Fig. 3A and B, traces a). In the presence of 0.4 mM phosphate, only a transient small potential was observed (Fig. 3A and B, traces b). In the presence of 12.5 μM OG and 0.4 mM phosphate, different effects where observed: in WT mitochondria, the ΔΨ was recovered (Fig. 3A, trace c), while in contrast, in the Δpor1 mitochondria OG failed to reestablish the ΔΨ (Fig. 3B, trace c). The fourth condition was the presence of 10 μM dVO_4 and 0.4 mM phosphate; here again, in WT mitochondria, dVO_4 addition resulted in a high ΔΨ (Fig. 3A, trace d), while in the Δpor1 strain, no ΔΨ was detected (Fig. 3B, trace d).

To further evaluate the sensitivity of the Δpor1 mitochondria to OG, the K^+-mediated swelling rates were compared in mitochondria from the WT and the Δpor1 strains. In either strain, the swelling rate was slow in the presence of 4 mM Pi (Fig. 4A and B, traces a) and it was fast in the presence of 0.4 mM Pi (Fig. 4A and B, traces b). In the presence of 12.5 μM OG, the rates of swelling were different depending on the source of mitochondria. In the WT mitochondria, OG inhibited the swelling rate observed at 0.4 mM Pi (Fig. 4A, trace c), while

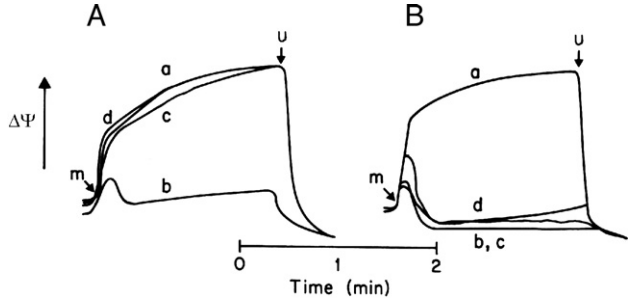

Fig. 3. Effect of Octilguanidine and decavanadate on the transmembrane potential of mitochondria isolated from a wild type or a Δpor1 strain of yeast. Experimental conditions: as in Fig. 1. (A)=mitochondria from the wild type strain, (B)=mitochondria from the Δpor1 strain. Both for panels A and B, traces were: a=4 mM Pi, b=0.4 mM Pi, c=0.4 mM Pi plus 12.5 μM OG, d=0.4 mM Pi plus 10 μM dVO_4. Representative experiment from $n=3$.

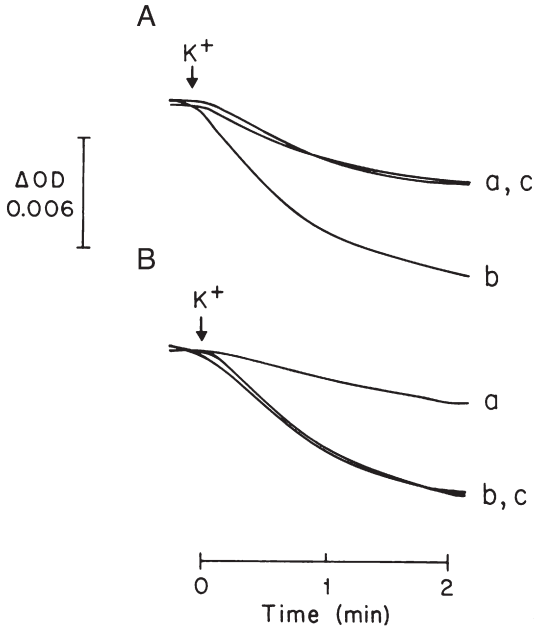

Fig. 4. Effect of Octilguanidine on the rate of swelling in mitochondria isolated from a wild type and a Δpor1 strain of yeast. Experimental conditions: as in Fig. 2, except no KCl was added in the medium. (A)=mitochondria from the wild type strain, (B)=mitochondria from the Δpor1 strain. Both for panels A and B, traces were: a=4 mM Pi, b=0.4 mM Pi, c=0.4 mM Pi plus 12.5 μM OG. K^+ indicates the addition of 20 mM KCl. Representative experiment from $n=3$.

in mitochondria from the Δpor1 strain, OG failed to inhibit the K^+-mediated swelling (Fig. 4B, trace c).

4. Discussion

The yeast mitochondrial unspecific channel (YMUC) and the mammalian permeability transition pore (PTP) share some features such as the approximate pore diameter, the depolarization ensuing upon opening and the mitochondrion swelling leading to mitochondrial dysfunction [1–6,13–17,19]; Nevertheless, these are not considered strictly equivalent [15]. In addition, procaspases induce a pattern of cell death in yeast which is similar to that observed in mammals [43,44]. The YMUC opens in the presence of high energy charge, promoting ATP hydrolysis by mitochondria, leading to suggest a role as an energy dissipation valve [16,17,45]. In addition, there is evidence that transient closing/opening (flickering) of permeability transition pores occurs in vivo [46]. Mitochondria located near the endoplasmic/sarcoplasmic reticulum are exposed to calcium "hotspots" which probably trigger flickering and the permeability transition [47,48]. Recent data suggest that PTP flickering modulates mitochondrial fusion and fission processes as well as cristae modifications [49]. Then, if this damage is considerable, the pore open state is fixed leading to mitochondrial degradation [50].

In yeast, pheromones promote increases in cytoplasmic calcium that last minutes. High cytoplasmic Ca^{2+} is observed both during mating and in different stages of the cell cycle [51,52]. In light of our data, high cytoplasmic Ca^{2+} would promote mitochondrial coupling thus enhancing energy production. There is also in vivo evidence that, at a high ATP/ADP ratio and low demand for ATP, opening of the YMUC dissipates the proton gradient, leading to ATP hydrolysis which promotes metabolic turnover [17].

Octylguanidine is a hydrophobic cation which binds to the mammalian PTP preventing opening [25], probably through interaction with Ca^{2+} binding sites. In this regard, it was observed that, in the mammalian heart, OG binding prevented Ca^{2+} effects such as the mitochondrial permeability transition and the mitochondrion-promoted death, both in cardiac myocytes and in the whole heart [25,26]. Both the YMUC and the mPTP are closed by OG and at least in yeast, this interaction is not dependent on $\Delta\Psi$. Such a strong binding has been explained by the stabilization of the OG–pore interaction resulting from the anchoring of the hydrophobic tail of OG in the membrane [21].

In mammalians, an external Me^{2+} ion binding site in the PTP has been reported to mediate pore closure with an IC_{50}=0.25 mM [20]. This value is relatively similar to the YMUC closure IC_{50} which is 0.3 mM [21]. These concentrations are similar to the ones used here; still, these are higher than the physiological concentrations of Ca^{2+} seen in yeast [51,52]. Nevertheless, when mating or reproducing, yeast exhibits Ca^{2+} transients that last up to 20 min [52], which perhaps explains the slow rate of Ca^{2+} uptake by yeast mitochondria [53].

Both the YMUC and the mPTP seem to possess cation-sensitive regulatory sites located in VDAC [21–24]. In this regard, in the mammalian VDAC, two Glu residues located on two different cytosolic VDAC loops have been proposed to interact specifically with Ca^{2+}. The point mutation of either of these Glu results in the loss of Ca^{2+} sensitivity of VDAC [23]. Yeast VDAC1 has low homology with its mammalian counterpart and thus it is difficult to assign possible binding sites, although it does possess a number of acid residues on predicted cytosolic loops (not shown).

In regard to the differential effect of dVO_4 on WT versus Δpor1 mitochondria, our results suggest that the dVO_4 also acts at the level of VDAC. These data are in agreement with reports on the inhibition of VDAC opening by polyanions [54,55].

Even in the absence of porin, the external mitochondrial membrane remains highly permeable, as suggested by the active swelling in response to K^+ and the preservation of the sensitivity to phosphate observed throughout this paper. Phosphate probably exerts its effects in the matrix of mitochondria [15,41]. This is also suggested by studies indicating that there is more than one highly conducting pore in the external membrane of mitochondria [3,4,56].

Both in yeast [17] and in mammalians [57], it has been demonstrated that VDAC is not needed for the permeability transition. Thus, the participation of VDAC in the yeast mitochondrial unspecific channel has been doubted, casting also second thoughts on whether the permeability pore of yeast and rat liver are equivalent [15]. Here, we demonstrate that, although the YMUC still exists in the absence of VDAC, the Ca^{2+}/OG/dVO_4-mediated control of pore opening is lost. This strongly suggests that VDAC regulates the pore. Furthermore, in a recently published model on the structure of the PTP, the

authors suggested that the open pore may not contain VDAC, spanning only the inner membrane, thus explaining the fact that solutes escaping the mitochondrial matrix invade the transmembrane space and not the cytoplasm [10]. Our results, indicating that VDAC is needed to close the pore but not to open it, are in agreement with the aforementioned model.

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